AVIAN DISEASES 47:319–329, 2003

Pathogenesis of Chicken-Passaged Newcastle Disease Viruses Isolated from Chickens and Wild and Exotic Birds

Glaucia D. Kommers,[A] Daniel J. King,[B] Bruce S. Seal,[B] and Corrie C. Brown[AC]

[A]Department of Pathology, College of Veterinary Medicine, University of Georgia, 501 D.W. Brooks Drive, Athens, GA 30602-7388

[B]Southeast Poultry Research Laboratory, U.S. Department of Agriculture, Agricultural Research Service, 934 College Station Road, Athens, GA 30605

Received 5 June 2002

SUMMARY. The pathogenesis of six Newcastle disease virus (NDV) isolates recovered from chickens (Ckn-LBM and Ckn-Australia) and wild (Anhinga) and exotic (YN parrot, pheasant, and dove) birds was examined after the isolates had been passaged four times in domestic chickens. Groups of 10 4-wk-old specific-pathogen-free white leghorn chickens were inoculated intraconjunctivally with each one of the isolates. The infected birds were observed for clinical disease and were euthanatized and sampled at selected times from 12 hr to 14 days postinoculation or at death. Tissues were examined by histopathology, by immunohistochemistry (IHC) to detect viral nucleoprotein (IHC/NP), and by *in situ* hybridization to detect viral mRNA and were double labeled for apoptosis (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling ([TUNEL] or IHC/caspase-3) and viral nucleoprotein (IHC/NP). Birds infected with the three low virulence viruses (Ckn-LBM, YN parrot, and Ckn-Australia) did not develop clinical disease. Microscopic lesions were observed only at the inoculation site and in organs of the respiratory system. The detection of viral nucleoprotein (N) was restricted to the inoculation site. The pheasant and dove isolates were highly virulent for chickens with marked tropism for lymphoid tissues, confirmed by the presence of large numbers of cells positive for viral N protein and viral mRNA. Viral N protein was detected early in the cytoplasm of cells in the center of the splenic ellipsoids. The apoptosis assays (TUNEL and IHC/caspase-3) showed increased apoptosis in the splenic ellipsoids as well. Apparently, apoptosis is an important mechanism in lymphoid depletion during NDV infection.

RESUMEN. Patogénesis de virus de la enfermedad de Newcastle replicados en pollo, obtenidos a partir de pollos y aves exóticas y salvajes.

Se examinó la patogénesis de seis aislados del virus de Newcastle obtenidos de pollos (Chk-LBM y Chk-Australia), aves salvajes (Anhinga) y aves exóticas (YN cotorra, faisán y paloma) luego de haberse realizado cuatro pasajes de los mismos en pollos domésticos. Se inocularon grupos de 10 aves del tipo leghorn, libres de patógenos específicos, de 4 semanas de edad mediante inyección en la conjuntiva ocular con cada uno de los aislados. Se observaron las aves infectadas para determinar la presencia de signos clínicos, y se realizaron varias tomas de muestras, después del sacrificio, en el periodo comprendido entre las 12 horas y 14 días después de la inoculación, o al momento del deceso de las aves. Las muestras de tejidos fueron examinadas mediante histopatología, mediante la técnica de immunohistoquímica para la detección de la nucleoproteína viral, mediante la técnica de hibridación *in situ* para la detección del ARN viral y fueron sometidas a un doble marcado para la detección de apoptosis (técnica de TUNEL, e hibridación *in situ* para caspasa 3) y la nucleoproteína viral. Las aves infectadas con los tres virus de baja virulencia (Chk-LBM, YN cotorra y Chk-Australia) no presentaron signos clínicos de enfermedad. Solo se observaron lesiones microscópicas en el sitio de la inoculación y en los órganos del sistema respiratorio. La detección de la nucleoproteína viral solo fue posible en el lugar de la inyección. Los aislados de faisán y paloma fueron altamente virulentos en los pollos en los cuales se observó un tropismo marcado del

[C]Corresponding author.

virus por el tejido linfoide, confirmado por la presencia de un gran número de células positivas a la presencia de la nucleoproteína y ARN viral. La nucleoproteína viral pudo ser detectada en las fases tempranas de la enfermedad en el citoplasma de las células de los centros de los elipsoides esplénicos. Las pruebas de apoptosis (TUNEL e hibridación *in situ* para caspasa 3) también demonstraron un aumento de la apoptosis en los elipsoides esplénicos. Aparentemente, la apoptosis es un mecanismo importante en la depleción de los tejidos linfoides durante la infección por el virus de Newcastle.

Key words: apoptosis, avian paramyxovirus-1, avian virology, chickens, double labeling, immunohistochemistry, *in situ* hybridization, Newcastle disease, pathogenesis, veterinary pathology

Abbreviations: APMV-1 = avian paramyxovirus type 1; BCIP = 5-bromo-4-chloro-3-indoylphosphate; BHI = brain–heart infusion; CALT = conjunctiva-associated lymphoid tissue; DPI = days postinoculation; EAC = ellipsoid-associated cell; GALT = gut-associated lymphoid tissue; HA = hemagglutination; HI = hemagglutination inhibition; HPI = hours postinoculation; ICPI = intracerebral pathogenicity index; IHC = immunohistochemistry; IHC/NP = immunohistochemistry to detect viral nucleoprotein; ISH = *in situ* hybridization; N = nucleoprotein; NBT = nitroblue tetrazolium; ND = Newcastle disease; NDV = Newcastle disease virus; SEPRL = Southeast Poultry Research Laboratory; SPF = specific-pathogen free; TUNEL = terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling; WL = white leghorn; YN = yellow nape

Newcastle disease (ND) is one of the most important avian viral diseases because of its economic impact on the poultry industry. Newcastle disease virus (NDV) is synonymous with avian paramyxovirus type 1 (APMV-1) (1,2) and has been classified in the order Mononegavirales, family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Rubulavirus* (1,2,21). However, some authors have proposed that NDV should have its own genus within the family *Paramyxoviridae* (22,28,33).

The clinical signs and gross or microscopic lesions observed in birds infected with NDV are not specific for ND. The clinical disease might range from subclinical infection to 100% mortality in a short period of time. Many factors related to the host (species, age, and immune status), virus strain (pathotype, dosage, and route of infection), and environmental or social stress can influence the severity and the course of the disease as well as the occurrence and distribution of the lesions (1,2,9).

When six NDV isolates recovered from chickens and exotic and wild birds were sequentially passaged in 2-wk-old white leghorn (WL) chickens by the intramuscular route of inoculation (13), the moderately virulent dove isolate became highly virulent for chickens. The originally highly virulent pheasant isolate had an increase in the intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index. Virulence increase was not observed during passages with the other four viruses studied (13). In the present study, the same six NDV isolates that were previously passaged in chickens (13) were inoculated in 4-wk-old WL chickens to assess clinical disease and pathogenesis after intraconjunctival inoculation, a more natural route of infection.

MATERIALS AND METHODS

Eggs and chickens. The source of embryonated chicken eggs and chickens was the Southeast Poultry Research Laboratory (SEPRL) specific-pathogen-free (SPF) WL flock. Embryonated eggs were utilized for virus amplification of chicken-passaged virus. Chickens were inoculated for the pathogenesis study and housed in negative-pressure isolators under BSL-3 agriculture conditions at SEPRL and provided feed and water *ad libitum* (3,12).

Viruses. Six chicken-passaged isolates of NDV were previously characterized by the OIE criteria for virulence as low virulence viruses and virulent viruses (13). The low virulence isolates were chicken–live bird market (Ckn–LBM) (APMV-1/chicken/U.S.[PA]/92-31003/92), a chicken isolate (ICPI = 0.00) from a live bird market in Pennsylvania, 1992; yellow nape parrot (YN parrot) (APMV-1/parrot/U.S.[TX]/96-22027/96), an isolate from a smuggled yellow nape parrot (ICPI = 0.24) in Texas, 1996; and Ckn-Australia (APMV-1/chicken/Australia/98-09-14-1110/98), a chicken isolate (ICPI = 0.05) from Australia, 1998.

The virulent isolates were anhinga (APMV-1/anhinga/U.S.[FL]/93-44083/93), isolated from an an-

hinga (ICPI = 1.31) at a commercial marine park in Florida, 1993; pheasant (APMV-1/pheasant/U.S./F98-1208/98), isolated from an exotic pheasant (ICPI = 1.88), 1998 (29); and Dove (APMV-1/dove/U.S./9248-10/98), isolated from an exotic dove (ICPI = 1.60) in a quarantine station, 1998. The reference velogenic viscerotropic Fontana strain (11) (APMV-1/chicken/U.S./CA1083[Fontana]/71; ICPI = 1.80) was inoculated in a group of birds for comparison. The isolates Ckn-LBM, YN parrot, anhinga, and dove were provided by the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, National Veterinary Services Laboratories, Ames IA. The Ckn-Australia isolate was received from Paul Selleck, Commonwealth Scientific and Industrial Research Organization, Australian Animal Health Laboratory at Geelong, Victoria, Australia.

Pathogenesis experiment. The viruses studied here were passaged four times in chickens as previously described (13). Briefly, three viruses (anhinga, pheasant, and dove) were passaged in 2-wk-old SPF WLs by intramuscular inoculation of infective spleen homogenates recovered from the previous passage. The other three viruses (Ckn-LBM, YN parrot, and Ckn-Australia) were passaged in 2-wk-old SPF WLs by a modified passage procedure. The inoculum was egg-amplified virus recovered from spleens or cloacal swabs from the previous passage and administered by the eyedrop/intranasal route.

Infective amnioallantoic fluid from the fourth chicken passage was prepared for inoculation into six groups of 10 4-wk-old SPF WLs. After dilution in brain–heart infusion (BHI) broth, approximately $10^{5.0}$ 50% embryo infective dose was inoculated intraconjunctivally (0.1 ml/bird). One group of 10 4-wk-old birds served as noninfected controls. With all six isolates, the disease was followed serially by examining tissues from birds euthanatized (two per day) at 2, 5, 10, and 14 days postinoculation (DPI) or at death.

With the pheasant and dove isolates, an early pathogenesis study was performed by intraconjunctival inoculation (0.1 ml/bird) of two groups of eight 4-wk-old chickens. Birds were euthanatized (two per day) at 12, 24, and 36 hr postinoculation (HPI) and at 2 DPI. One group of eight 4-wk-old birds was infected with the Fontana strain for comparison. One group of eight 4-wk-old birds served as noninfected controls.

Necropsies were performed immediately postmortem and the following tissues were collected and fixed by immersion in 10% neutral buffered formalin for approximately 48–52 hr: spleen, thymus, bursa, lower eyelid (including conjunctiva and skin), Harderian gland, esophagus, ingluvium, proventriculus, pancreas, small intestine, cecal tonsils, large intestine, caudal thoracic air sac, breast and thigh muscles, comb, trachea, lung, heart, liver, kidney, brain, turbinates (including nasal mucosa), and bone marrow (femur). The sections of femur and turbinates were decalcified in 5% formic acid for 3–4 hr. All sampled tissues were routinely processed into paraffin, and 3-μm sections were cut for hematoxylin and eosin staining, immunohistochemistry (IHC), *in situ* hybridization (ISH), and apoptosis assays.

Virus isolation, titration, and hemagglutination–inhibition (HI) test. Immediately prior to euthanasia, oropharyngeal (oral) and cloacal swabs were obtained from each bird and placed in a tube containing 1.5 ml of BHI with antibiotics (2000 units/ml penicillin G, 200 μg/ml gentamicin sulfate, and 4 μg/ml amphotericin B; Sigma Chemical Co., St. Louis, MO). Swab fluids were centrifuged at 1000 × *g* for 20 min, and undiluted supernatant was inoculated into 9-to-10-day-old SPF embryonated chicken eggs and incubated for 7 days. Virus infectivity titers of inoculum during the experiments were calculated from the results of inoculation of 9- or 10-day-old embryonated eggs with serial 10-fold dilution in BHI containing antibiotics (100 units penicillin G/ml and 50 μg gentamicin sulfate/ml). NDV-infected dead or surviving embryos were identified by hemagglutination (HA) activity in amniotic–allantoic fluid harvested from chilled eggs. NDV was confirmed in HA-positive samples by the HI test with NDV-specific antiserum (10). At 10 and 14 DPI, birds were bled immediately prior to euthanasia. The infection of all inoculated birds was confirmed by virus isolation or by seroconversion (HI test). The HI test was conducted by conventional microtiter methods (10).

IHC. All sampled tissues were examined by IHC by the following protocol to detect viral nucleoprotein (IHC/NP). After deparaffinization, tissue sections were subjected to antigen retrieval by microwaving for 10 min at full power in Vector antigen unmasking solution (Vector Laboratories, Burlingame, CA) followed by blocking with universal blocking reagent (Biogenex, San Ramon, CA) as recommended by the manufacturer. Incubation with the primary antibody, an anti-peptide antibody made in rabbit (12) and used at 1:8000 dilution, was overnight at 4 C or for 2 hr at 37 C. After washing, sections were incubated with biotinylated antibody against the species in which the primary antibody was made and then with avidin–biotin–alkaline phosphatase (Vector Laboratories). Substrate was Vector Red (Vector Laboratories). Sections were counterstained lightly with hematoxylin and coverslipped with Permount for a permanent record.

Double labeling for detection of apoptosis and viral protein. The TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling) assay (24) (Boehringer Mannheim, Indianapolis, IN) was utilized to detect apoptosis in sections of lymphoid organs (spleen,

bursa, and thymus) of Fontana-, pheasant-, and dove-infected birds. The tissue sections were microwaved for 10 min at full power in Vector antigen unmasking solution (Vector Laboratories). After washing, the sections were incubated with TUNEL reaction mixture for 1 hr at 37 C. Converter-AP was added to the slides for 30 min at 37 C. The development was with chromogen/substrate nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoylphosphate (BCIP). After the TUNEL assay was performed, the tissue sections were washed and stained by the IHC/NP protocol as described above, starting at the blocking step. Sections were counterstained lightly with hematoxylin and coverslipped with Permount for a permanent record.

Sections of the spleen were also double labeled by two sequential rounds of IHC to detect apoptotic cells (IHC/caspase-3) and viral N protein (IHC/NP). In the first round of IHC, deparaffinized spleen sections were treated with 3% hydrogen peroxide and then subjected to antigen retrieval followed by blocking as described in the IHC/NP protocol. Incubation with the primary antibody, a rabbit polyclonal anti-active caspase-3 antibody (Promega, Madison, WI) at 1:350 dilution (14), was for 2 hr at 37 C. After washing, sections were incubated with biotinylated antibody against the species in which the primary antibody was made and then with elite-PO (Vector Laboratories). Substrate was diaminobenzidine (Vector Laboratories). After the IHC/caspase-3 protocol was performed, tissue sections were washed and a second round of IHC was performed by the IHC/NP protocol as described above, starting at the incubation with the primary antibody. Sections were counterstained lightly with hematoxylin and coverslipped with Permount for a permanent record.

ISH. Selected tissue sections of birds infected with three of the virulent viruses (pheasant, dove, and Fontana) were stained with a negative-sense digoxigenin-labeled 850-base riboprobe representing the 5′ end of the matrix gene of NDV Fontana (CA 1083) strain as previously described (3,12,14). The matrix gene from the Fontana strain was cloned into pCRII transcription vectors (Invitrogen, Carlsbad, CA). Anti-sense digoxigenin-labeled riboprobes were generated with RNA polymerase in the presence of labeled nucleotides. For ISH, tissue sections were deparaffinized, rehydrated, and digested with 30 μg/ml proteinase K for 15 min at 37 C. Hybridization was conducted overnight at 42 C with approximately 20 ng of probe in prehybridization solution. After stringent washes, anti-digoxigenin alkaline phosphatase was added to the sections. The development was with chromogen/substrate NBT/BCIP. Tissues were counterstained lightly with hematoxylin and coverslipped.

RESULTS

Clinical *signs.* *Low virulence viruses.* Neither clinical signs nor mortality was observed in chickens inoculated with the Ckn-LBM, YN parrot, and Ckn-Australia isolates.

Virulent viruses. Neither clinical signs nor mortality was observed in the anhinga-infected birds. In the early pathogenesis study, clinical signs were not observed in birds infected with pheasant and dove isolates during the first 36 HPI. A few Fontana-infected birds had ruffled feathers at 36 HPI, and birds had ruffled feathers and hunched posture at 2 DPI. Mortality was not observed in birds infected with the Fontana strain during the 2-day observation period, and all birds were euthanatized at that time. With the pheasant isolate at 2 DPI, all birds were slightly depressed and a few had head twitching. At 3 DPI, all birds were depressed, with diarrhea, and four birds had reddened and swollen conjunctiva. All the remaining birds were found dead at 4 DPI. With the dove isolate, all birds were slightly depressed with ruffled feathers at 3 DPI. At 4 DPI, all birds were very depressed. Five birds were found dead at 5 DPI. One remaining bird was very depressed. Clinical signs were not observed in the noninfected controls.

Gross findings. *Low virulence viruses.* At 2 DPI, the infected birds had enlarged and dark spleens and eyelid petechiation. At 5 DPI, there were slight reddening of the eyelids, mild pulmonary edema, and slight petechiation in the thymus. At 10 DPI and 14 DPI, a few petechiae were observed in the eyelids of all infected birds.

Virulent viruses. At 2 DPI, chickens infected with the moderately virulent anhinga isolate had petechiae (eyelids and thymus), multifocal hemorrhages (the thigh muscles), and small spleens. At 5 DPI, infected birds had pulmonary edema and hemorrhages on the thigh muscles. Petechiae in the eyelids (10 and 14 DPI) and crusty dark spots on the comb tips (10 DPI) were also observed.

In the early pathogenesis study with the highly virulent pheasant, dove, and Fontana isolates, infected birds had reddening and petechiae in the eyelids at 12 HPI and foci of reddening at the cecal tonsils and fluid intestinal contents at 24 HPI. Petechiae and edema in the eyelids, dark red foci at the serosal surface of the cecal tonsils, fluid intestinal contents, and large/mottled spleens were observed at 36 HPI and 2 DPI.

Widespread gross lesions were observed in the chickens infected with both pheasant (4 DPI) and dove (4 and 5 DPI) isolates. There were eyelid edema and reddening, dehydration, muscle wasting, subcutaneous neck edema, pale/mottled spleens, small/edematous thymus, small bursa, pale bone marrow, and edematous pancreas. Multifocal hemorrhages at the opening of the mucosal glands was a very common finding in the proventriculus. Other findings were petechiation in the cloaca and reticulate kidneys (interpreted to be urate deposits).

Histopathology. *Low virulence viruses.* Pulmonary edema was detected at 10 and 14 DPI in the Ckn-LBM–infected birds. In YN parrot–infected birds, lymphoplasmacytic conjunctivitis and tracheitis were observed at 2, 5, and 10 DPI. There was lymphoplasmacytic and hyperplastic airsacculitis at 10 (see Fig. 1) and 14 DPI. Australia-infected birds had lymphoplasmacytic conjunctivitis (2, 5, and 10 DPI) and lymphoplasmacytic and hyperplastic tracheitis (5, 10, and 14 DPI).

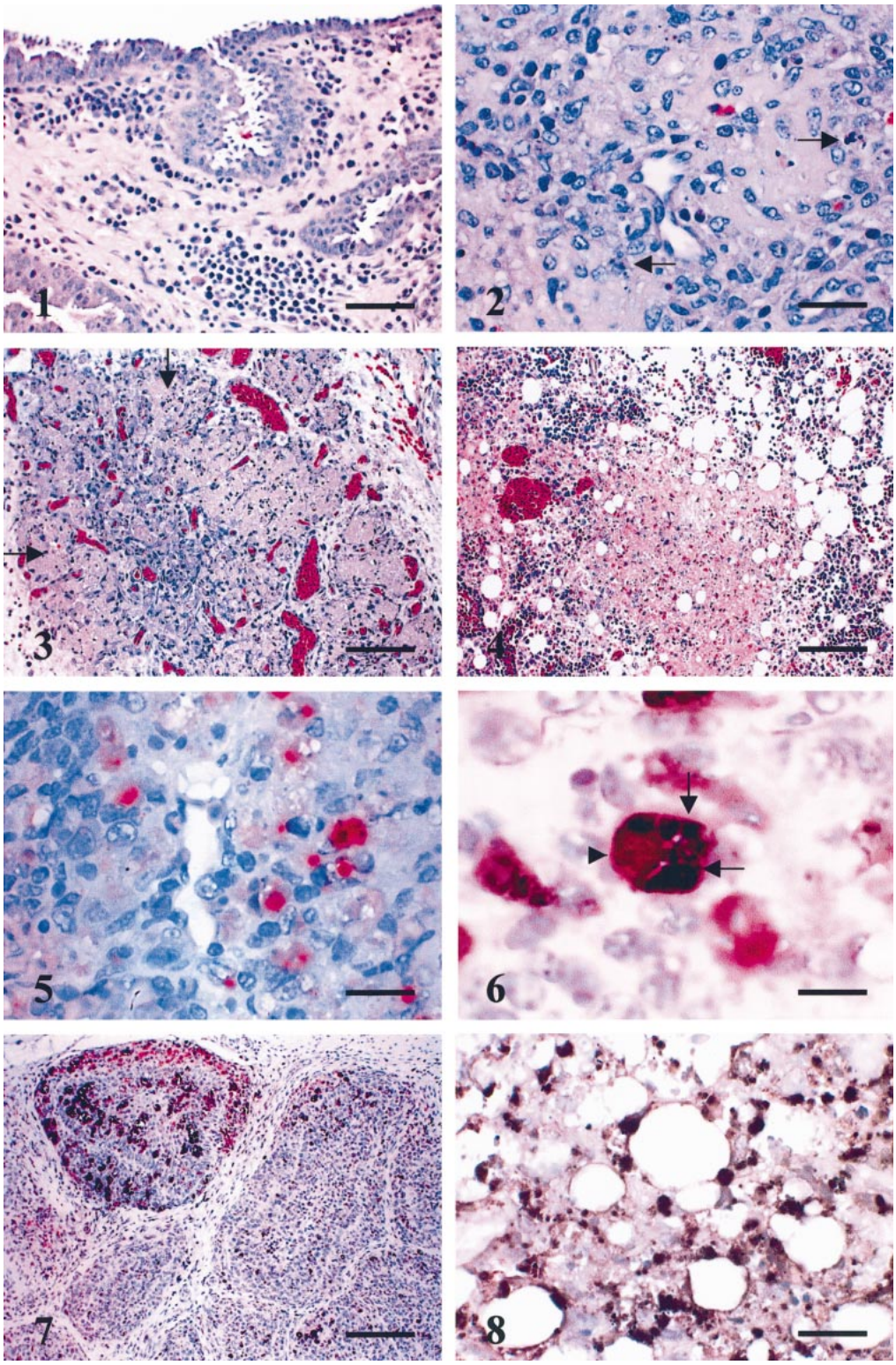
Virulent viruses. The microscopic findings observed in birds infected with the anhinga, pheasant, dove, and Fontana isolates are summarized in Table 1. With the anhinga isolate, the main lesions observed were in the brain, heart, and comb tips. In the pheasant- and dove-infected birds, the main lesions observed were in the lymphoid tissues and were characterized by apoptosis, depletion, and lymphocellular necrosis, as illustrated in Figs. 2, 3. Severe bone marrow necrosis was another consistent finding (Fig. 4). In birds inoculated with the Fontana strain, the early microscopic findings observed were in the nasal mucosa, cecal tonsils, conjunctiva-associated lymphoid tissue (CALT), splenic ellipsoids, thymus, bursal follicles, gut-associated lymphoid tissue (GALT), and bone marrow. Microscopic lesions were not found in the noninfected controls.

IHC/NP. *Low virulence viruses.* There were a few IHC/NP-positive epithelial cells in the conjunctiva at 2 (with YN parrot and Ckn-Australia isolates) and 5 DPI (with Ckn-LBM, YN parrot, and Ckn-Australia isolates).

Virulent viruses. With the anhinga isolate, IHC/NP-positive staining was detected only at 5 DPI in the cardiac myofibers and in the comb epithelium. Detectable amounts of viral N protein, consisting of a few positive cells in the lymphoid aggregates (eyelids and nasal mucosa), were observed in Fontana-infected birds at 36 HPI. In dove-infected birds at 36 HPI, a few weakly positive cells were observed in the nasal epithelium. In chickens infected with the pheasant and dove isolates, the most striking staining was observed in multiple organs at 2 (Fig. 5), 4, and 5 DPI. The lymphoid organs and lymphoid aggregates (mostly GALT) were the tissues with the largest amounts of viral N protein at 4 and 5 DPI. The IHC results for 2, 4, and 5 DPI are summarized in Table 2 (only positive tissues are listed). The tissues of the noninfected controls were negative by the IHC/NP assay.

Double labeling. The double-labeled sections of the lymphoid organs of Fontana-, pheasant-, and dove-infected birds stained with the combined TUNEL-IHC/NP assays revealed similar results for all three isolates. In sections of the spleen, slightly to moderately increased numbers of apoptotic cells (TUNEL-positive) were observed mostly surrounding the penicilliform capillaries in the ellipsoids at 2 DPI. Most of the TUNEL-positive cells were negative for viral N protein at that time, but were located in the neighborhood of IHC/NP-positive cells. A few double-labeled cells, characterized by black-stained nuclei (TUNEL positive) and red-stained cytoplasm (IHC/NP positive), were seen mostly in the ellipsoids at 2 DPI (Fig. 6). At 4 and 5 DPI, numerous widespread splenic cells were positive either by IHC/NP or the TUNEL reaction. Double-labeled cells were more often seen and were randomly distributed at that time. In the noninfected controls, rare randomly distributed TUNEL-positive cells were observed in the spleen sections, and all cells were negative for viral N protein.

In sections of the bursa at 2 DPI, slightly increased numbers of TUNEL-positive cells were observed, mostly in the cortex of the follicles. Scarce N protein–positive cells were observed mostly in the medulla at this time. In later infection (4 and 5 DPI), the number of apoptotic cells was remarkably increased, especially in some follicles that were also more strongly positive for viral N protein than in others, as observed in Fig. 7. Double-labeled cells were occasionally observed. In the noninfected controls, a small number of TUNEL-positive cells were detected, mostly in the fol-


1
2
3
4
5
6
7
8

licular cortex, and all cells were negative for viral N protein.

In sections of the thymus at 2 DPI, slightly increased numbers of TUNEL-positive cells were observed in the cortex. Viral N protein was detected in a few medullary cells at 2 DPI. There were apoptotic cells in the vicinity of the N protein–positive cells. In some birds, the presence of TUNEL-positive and N protein–positive cells was more striking in some thymic lobes than in others after 2 DPI. Scattered TUNEL-positive cells were observed, mostly in the cortex of noninfected controls. All cells were negative for viral N protein in the controls.

With the combined IHC/caspase-3 and IHC/NP in spleen sections, increased number of apoptotic cells (brown-stained cytoplasm) were observed in the ellipsoids at 2 DPI and were located close to N protein–positive cells (red-stained cytoplasm). The morphology (dendrite shaped) and location of several IHC/caspase-3–positive cells were compatible with the ellipsoid-associated cells (EACs). Double-labeled cells were rarely observed by this method. Caspase-3–positive cells were seen in small numbers and were randomly distributed in the spleen sections of the noninfected controls. Viral N protein was not detected in the controls.

ISH. The selected tissue sections of birds infected with Fontana, pheasant, and dove isolates stained by ISH confirmed the distribution of the viral infection detected by IHC/NP. The use of ISH was slightly more sensitive for detecting early viral infection during the pathogenesis study (first 2 DPI) than IHC/NP. More striking ISH-positive staining was observed at 2, 4 (Fig. 8), and 5 DPI. The tissues of the noninfected controls were negative by the ISH assay.

DISCUSSION

This study was performed to assess clinical disease and pathogenesis of six chicken-passaged NDV isolates. Birds infected with three low virulence viruses (Ckn-LBM, YN parrot, and Ckn-Australia) did not develop clinical disease after intraconjunctival inoculation. Histologic lesions were observed only in the inoculation site and in organs of the respiratory system. However, the detection of viral nucleoprotein (N) in affected organs was restricted to the inoculation site. It is possible that the virus load in the infected cells of other affected tissues was too low to be detected by IHC. Previous studies have demonstrated minimal positive staining detected by ISH with two low virulence reference strains (B1 and QV4) (3).

Among the virulent viruses, the anhinga isolate showed moderate virulence for chickens after intraconjunctival inoculation. Although microscopic lesions were detected in the brain,

←

Fig. 1. Epithelial hyperplasia and multifocal lymphoplasmacytic and histiocytic infiltrates in the air sac of a chicken infected with the YN parrot isolate at 10 days postinfection. Hematoxylin and eosin. Bar = 60 μm.

Fig. 2. Apoptotic cells (arrows) and lymphoid cell depletion in the splenic ellipsoids of a chicken infected with the pheasant isolate at 2 days postinfection. Hematoxylin and eosin. Bar = 40 μm.

Fig. 3. Severe diffuse necrosis (arrows) of the thymus of a chicken infected with the dove isolate at 5 days postinfection. Hematoxylin and eosin. Bar = 90 μm.

Fig. 4. Severe multifocal necrosis of the bone marrow (femur) of a chicken infected with the dove isolate at 4 days postinfection. Hematoxylin and eosin. Bar = 90 μm.

Fig. 5. Viral nucleoprotein–positive cells (red stained) in the splenic ellipsoids of a chicken infected with the dove isolate at 2 days postinfection. IHC, avidin–biotin–alkaline phosphatase, hematoxylin counterstain. Bar = 40 μm.

Fig. 6. Double-labeled cell with a red-stained cytoplasm (nucleoprotein positive; arrowhead) and an apoptotic dark-stained nucleus (TUNEL positive; arrows) in the splenic ellipsoids of a chicken infected with the pheasant isolate at 2 days postinfection. TUNEL assay and IHC (avidin–biotin–alkaline phosphatase), hematoxylin counterstain. Bar = 10 μm.

Fig. 7. Large number of apoptotic cells (dark-stained nuclei) in a bursal follicle where viral nucleoprotein–positive cells (red-stained cytoplasm) are also observed. Chicken infected with the dove isolate at 4 days postinfection. TUNEL assay and IHC (avidin–biotin–alkaline phosphatase), hematoxylin counterstain. Bar = 90 μm.

Fig. 8. Numerous cells (dark stained) are positive for viral mRNA in the bone marrow (femur) of a chicken infected with the pheasant isolate at 4 days postinfection. ISH, hematoxylin counterstain. Bar = 60 μm.

Table 1. Microscopic findings in organs of birds infected with four virulent NDV isolates (anhinga, pheasant, dove, and Fontana).

Isolate	Time postinoculation[A]	Lesions[B]
Anhinga	2, 5 DPI	Mild lymphoplasmacytic encephalitis
	5, 10 DPI	Lymphoplasmacytic tracheitis; pustules (comb tips)
	5, 10, 14 DPI	Multifocal myocardial necrosis; lymphohistiocytic myocarditis
	10 DPI	Necrosis of Purkinje cells and other neurons, neuronophagia, gliosis, and perivascular cuffing (cerebrum and cerebellum)
Pheasant	36 HPI	Lymphoplasmacytic conjunctivitis; mucous rhinitis; lymphoid depletion (cecal tonsils)
	2 DPI	Increased apoptosis (splenic ellipsoids, thymus, GALT, cecal tonsils) and lymphoid depletion (CALT, bursa, thymus) and lymphocellular necrosis (CALT); conjunctivitis with epithelial necrosis; hyperplastic and mucous rhinitis; necrotizing and lymphoplasmacytic laryngitis; lymphoplasmacytic tracheitis; and bone marrow necrosis
	4 DPI	Severe diffuse necrosis of all lymphoid (with fibrin deposits and heterophils) and hematopoietic tissues; conjunctivitis; necrosis and hemorrhage of the proventricular glands; hemorrhages in the guts; pancreatic necrosis; necrotizing and lymphocytic myocarditis; cerebellar gliosis; and pustules (comb tips)
Dove	24 HPI	Mild conjunctivitis; multifocal hemorrhages (nasal mucosa)
	36 HPI	Mild multifocal necrosis and apoptosis (splenic ellipsoids); increased apoptosis (thymus, bursal follicles, GALT, cecal tonsils); lymphoplasmacytic conjunctivitis, laryngitis, and tracheitis
	2 DPI	Increased apoptosis (splenic ellipsoids and germinal centers, bursa, thymus, cecal tonsils); lymphoplasmacytic conjunctivitis, tracheitis, and rhinitis
	4, 5 DPI	Severe diffuse necrosis of all lymphoid (with fibrin deposits and heterophils) and hematopoietic tissues; pustules (comb); necrosis of osteoclasts (femur)
Fontana	24 HPI	Edema and hemorrhage (nasal mucosa); multifocal hemorrhages (cecal tonsils)
	36 HPI	Increased apoptosis (CALT, thymus, bursal folicles); multifocal hemorrhages and lymphoid depletion (cecal tonsils); pulmonary congestion and edema; lymphoplasmacytic rhinitis, laryngitis, and airsacculitis
	2 DPI[C]	Eyelid edema and conjunctivitis; increased apoptosis and mild necrosis (splenic ellipsoids); mild lymphoid depletion and necrosis (GALT, cecal tonsils); mild multifocal necrosis (bone marrow)

[A]DPI = days postinoculation; HPI = hours postinoculation.
[B]GALT = gut-associated lymphoid tissue; CALT = conjunctiva-associated lymphoid tissue.
[C]All birds were euthanatized at 2 DPI.

heart, trachea, and comb, viral N protein was detected by IHC only in the heart and comb. The absence of detectable viral N protein in the brain might be because the most remarkable brain lesions occurred late (at 10 and 14 DPI), when NDV is rarely detected (3,34). Brown *et al.* (3), working with the original anhinga isolate inoculated intraconjunctivally in chickens, detected viral mRNA by ISH only in the spleen (5 DPI) and heart (5 and 10 DPI). However, during a passage study (13) with this virus, viral N protein was detected in the eyelids, heart, trachea, comb, and brain of chickens inoculated intramuscularly. This fact shows that the route of inoculation may also influence the sites of viral replication, as previously suggested for NDV (3).

The pheasant and dove isolates were highly virulent for chickens during the early pathogenesis study (first 48 HPI) when compared with the highly virulent Fontana strain, a psittacine-origin virus that caused the California viscerotropic velogenic ND outbreaks in chickens in the early 1970s (32). Although the Fontana-infected birds presented clinical signs earlier than the pheasant- and dove-infected birds, mild gross lesions were observed at the inoculation site at 12 HPI and microscopic lesions

Table 2. Extent[A] of viral infection characterized by the presence of NDV nucleoprotein (N) detected by IHC in tissues from chickens inoculated with three different highly virulent NDV isolates (pheasant, dove, and Fontana).

	Pheasant[B]		Dove			Fontana
Affected tissue	2 DPI	4 DPI	2 DPI	4 DPI	5 DPI	2 DPI
Comb	−	+	−	+	+	−
Eyelid	+	+++	+	++	++	+
Harderian gland	+	−	−	−	+	−
Bursa	+	+++	−	+	++	−
Cecal tonsils	++	+++	+	+++	+++	+
Spleen	+	+++	+	++	++	+
Thymus	+	+++	+	+++	+++	+
Bone marrow	+	+++	+	+++	+++	−
Tongue	−	−	−	−	+	−
Salivary gland	+	+	−	+	+	−
Crop	+	++	−	+	+	−
Esophagus	−	−	−	−	+	−
Proventriculus	+	++	+	++	++	−
Intestines	++	++	+	+++	+++	−
Pancreas	−	−	−	−	+	−
Kidney	+	++	−	+	+++	−
Nasal turbinates	−	NS[C]	+	NS	NS	+
Larynx	++	+++	+	++	−	−
Trachea	−	+	−	+	+	−
Air sac	−	−	−	−	+	−
Lung	+	++	−	+	++	−
Heart	−	+	−	−	+	−
Brain	+	+	−	+	+	−

[A]− = negative for NDV (N); + = small amount of N; ++ = moderate amount of N; +++ = large amount of N.

[B]All infected birds were dead by 4 DPI.

[C]NS = not sampled.

were first observed at 24 HPI with all three of these viruses.

Viral N protein and mRNA were first detected (at 36 HPI) in large mononuclear cells in the CALT and in lymphoid aggregates of the nasal mucosa/turbinates of Fontana- and dove-infected birds. It seems that the next areas infected after intraconjunctival inoculation of these highly virulent NDV isolates were the lymphoid aggregates of the nasal mucosa/turbinates. This result might suggest the involvement of macrophages in the replication and dissemination of NDV. Brown *et al.* (3) reported evidence of extensive viral replication within macrophages with subsequent spread to many organs (mostly lymphoid tissues) in birds infected with the viscerotropic velogenic pathotype. An *in vitro* study reported that chicken macrophages support the growth and replication of NDV. Intact virus particles were also observed by electron microscopy in macrophages exhibiting features of apoptosis (18).

On the basis of the number of organs positive for viral N protein or mRNA at 2 DPI, detected by IHC or ISH, respectively, the pheasant isolate spread more rapidly throughout body tissues than the Fontana and dove isolates. Disseminated gross and microscopic lesions in the lymphoid tissues showed marked lymphotropism, as previously described with other highly virulent NDV isolates (3,12,15,25). The lymphotropism was confirmed by the presence of large numbers of cells positive for viral N protein and viral mRNA (detected by IHC and ISH, respectively) in all lymphoid tissues. An interesting finding was the presence of large amounts of viral antigen and mRNA in areas where the vast majority of the cells are lymphocytes, suggesting that these cells are also permissive to viral replication *in vivo,* as reported *in vitro* (16).

Another prominent finding in our study was the presence of large amounts of IHC/NP-positive and viral mRNA-positive cells in the bone marrow (femur) of birds infected with the pheasant and dove isolates. However, the presence of infected cells in the skeletal muscles examined was negligible. These findings are important when considering trade of fresh poultry meat from countries where ND has been observed in commercial poultry.

Although the ability of NDV to induce apoptosis and lysis of several types of neoplastic cells has been the focus of many cancer therapy studies (4,5,6,23,30), a relatively limited number of studies have reported the importance of NDV-induced apoptosis in avian infected cells or tissues (14,16,17,18,19,20,27). In this experiment, an increased number of apoptotic cells was observed microscopically in the splenic ellipsoids, mostly at 2 DPI. The splenic ellipsoids and especially the EACs (antigen-presenting cells) are the first localization of several different kinds of antigens, including infectious agents, in the spleen of chickens (7). The apoptosis assays (TUNEL and IHC/caspase-3) confirmed the increased number of apoptotic cells in the ellipsoids at 2 DPI, and several IHC/caspase-3–positive apoptotic cells had the cytoplasmic morphology of the EACs, as previously described with other APMV-1 and PPMV-1 isolates (14). Viral N protein was also detected in the cytoplasm of cells in the center of the ellipsoids. Double-labeled cells were better visualized by the combined TUNEL-IHC/NP. These results along with the finding of increased numbers of apoptotic cells, especially in the bursal follicles or thymic lobes where N protein was more abundantly detected, confirm that apoptosis is an important mechanism in lymphoid depletion during NDV infection. Similar results were reported with other avian viral diseases including highly pathogenic avian influenza (26) and infectious bursal disease virus in chickens (8,31). NDV-induced apoptosis appears to be due to both direct and indirect effects of viral infection. The simultaneous positive staining for apoptosis and viral N protein is indicative of a direct effect of NDV causing apoptosis of the infected cells. Indirect effects have been suggested previously for NDV (30) and for other avian viral agents (8,26,31) and are presumed related to release of cytokines by the infected cells.

REFERENCES

1. Alexander, D. J. Newcastle disease and other Paramyxoviridae infections. In: Diseases of poultry, 10th ed. B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald, and Y. M. Saif, eds. Iowa State University Press, Ames, IA. pp. 541–569. 1997.

2. Alexander, D. J. Newcastle disease virus and other paramyxoviruses. In: A laboratory manual for the isolation and identification of avian pathogens, 4th ed. D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 156–163. 1998.

3. Brown, C., D. J. King, and B. S. Seal. Pathogenesis of Newcastle disease in chickens experimentally infected with viruses of different virulence. Vet. Pathol. 36:125–132. 1999.

4. Cassel, W. A., and R. E. Garret. Newcastle disease virus as an antineoplastic agent. Cancer 18: 863–868. 1965.

5. Csatary, L. K., and T. Bakacs. Use of Newcastle disease virus vaccine (MTH-68/H) in a patient with high-grade glioblastoma. J. Am. Med. Assoc. 281:1588–1589. 1999.

6. Fabian, Z., B. Torocsik, K. Kiss, L. K. Csatary, B. Bodey, J. Tigyi, C. Csatary, and J. Szeberenyi. Induction of apoptosis by a Newcastle disease virus vaccine (MTH-68/H) in PC12 rat phaeochromocytoma cells. Anticancer Res. 21:125–135. 2001.

7. Jeurissen, S. H. M. Structure and function of the chicken spleen. Res. Immunol. 142:352–355. 1991.

8. Jungmann, A., H. Nieper, and H. Müller. Apoptosis is induced by infectious bursal disease virus replication in productively infected cells as well as in antigen-negative cells in their vicinity. J. Gen. Virol. 82:1107–1115. 2001.

9. Kaleta, E. F., and C. Baldauf. Newcastle disease in free-living and pet birds. In: Newcastle disease. D. J. Alexander, ed. Kluwer Academic Publishers, Boston, MA. pp. 197–246. 1988.

10. King, D. J. Influence of chicken breed on pathogenicity evaluation of velogenic neurotropic Newcastle disease virus isolates from cormorants and turkeys. Avian Dis. 40:210–217. 1996.

11. King, D. J. Avian paramyxovirus type 1 from pigeons: isolate characterization and pathogenicity after chicken or embryo passage of selected isolates. Avian Dis. 40:707–714. 1996.

12. Kommers, G. D., D. J. King, B. S. Seal, and C. C. Brown. Virulence of pigeon-origin Newcastle disease virus isolates for domestic chickens. Avian Dis. 45:906–921. 2001.

13. Kommers, G. D., D. J. King, B. S. Seal, and C. C. Brown. Virulence of six heterogeneous-origin Newcastle disease virus isolates before and after se-

quential passages in domestic chickens. Avian Pathol. In press.

14. Kommers, G. D., D. J. King, B. S. Seal, K. P. Carmichael, and C. C. Brown. Pathogenesis of six pigeon-origin isolates of Newcastle disease virus for domestic chickens. Vet. Pathol. 39:353–362. 2002.

15. Kuiken, T., G. Wobeser, F. A. Leighton, D. M. Haines, B. Chelack, J. Bogdan, L. Hassard, R. A. Heckert, and J. Riva. Pathology of Newcastle disease in double-crested cormorants from Saskatchewan, with comparison of diagnostic methods. J. Wildl. Dis. 35:8–23. 1999.

16. Lam, K. M. Newcastle disease virus–induced apoptosis in the peripheral blood mononuclear cells of chickens. J. Comp. Pathol. 114:63–71. 1996.

17. Lam, K. M. Ultrastructural changes in the cardiac muscle of chickens infected with the GB strain of Newcastle disease virus. J. Comp. Pathol. 114:73–79. 1996.

18. Lam, K. M. Growth of Newcastle disease virus in chicken macrophages. J. Comp. Pathol. 115:253–263. 1996.

19. Lam, K. M., and A. C. Vasconcelos. Newcastle disease virus–induced apoptosis in chicken peripheral blood lymphocytes. Vet. Immunol. Immunopathol. 44:45–56. 1994.

20. Lam, K. M., A. C. Vasconcelos, and A. A. Bickford. Apoptosis as a cause of death in chicken embryos inoculated with Newcastle disease virus. Microb. Pathog. 19:169–174. 1995.

21. Lamb, R. A., and D. Kolakofsky. Paramyxoviridae: the viruses and their replication. In: Fields virology, 3rd ed. B. N. Fields, D. M. Knipe, and P. M. Howley, eds. Lippincott-Raven Publ., Philadelphia, PA. pp. 1177–1204. 1996.

22. Leeuw, O., and B. Peeters. Complete nucleotide sequence of Newcastle disease virus: evidence of a new genus within the subfamily Paramyxovirinae. J. Gen. Virol. 80:131–136. 1999.

23. Lorence, R. M., P. A. Rood, and K. W. Kelley. Newcastle disease virus as an antineoplastic agent induction of tumor necrosis factor-alpha and augmentation of its cytotoxicity. J. Natl. Cancer Inst. 80: 1305–1312. 1988.

24. Negoescu, A., P. Lorimier, F. Labat-Moleur, C. Drouet, C. Robert, C. Guillermet, C. Brambilla, and E. Brambilla. In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparation. J. Histochem. Cytochem. 44:959–966. 1996.

25. Ojok, L., and C. Brown. An immunohistochemical study of the pathogenesis of virulent viscerotropic Newcastle disease in chickens. J. Comp. Pathol. 115:221–227. 1996.

26. Perkins, L. E. L., and D. E. Swayne. Pathobiology of A/Chicken/Hong Kong/220/97 (H5N1) avian influenza in seven gallinaceous species. Vet. Pathol. 38:149–164. 2001.

27. Rautenschlein, S., and J. M. Sharma. Response of turkeys to simultaneous vaccination with hemorrhagic enteritis and Newcastle disease viruses. Avian Dis. 43:286–292. 1999.

28. Seal, B. S., D. J. King, and R. J. Meinersmann. Molecular evolution of the Newcastle disease virus matrix protein gene and phylogenetic relationship among the Paramyxoviridae. Virus Res. 66:1–11. 2000.

29. Shivaprasad, H. L., D. Rupiper, P. R. Woolcock, and L. Woods. An outbreak of Newcastle disease in exotic pheasant and doves. In: Proc. 48th Annual Western Poultry Conference, Vancouver, Canada. p. 43. 1999.

30. Sinkovics, J. G., and J. C. Horvath. Newcastle disease virus (NDV): brief history of its oncolytic strains. J. Clin. Virol. 16:1–15. 2000.

31. Tanimura, N., and J. M. Sharma. In-situ apoptosis in chickens infected with infectious bursal disease virus. J. Comp. Pathol. 118:15–27. 1998.

32. Utterback, W. W., and J. H. Schwartz. Epizootiology of velogenic Newcastle disease in southern California, 1971–1973. J. Am. Vet. Med. Assoc. 63: 1080–1088. 1973.

33. Westover, K. M., and A. L. Hughes. Molecular evolution of viral fusion and matrix protein genes and phylogenetic relationships among the Paramyxoviridae. Mol. Phylogenet. Evol. 21:128–134. 2001.

34. Wilczynsky, S. P., M. L. Cook, and J. G. Stevens. Newcastle disease as model for paramyxovirus-induced neurologic syndromes. II. Detailed characterization of the encephalitis. Am. J. Pathol. 89:649–666. 1977.

ACKNOWLEDGMENTS

G. D. Kommers was supported by a scholarship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. The work was funded by U.S. Poultry and Egg Association grant 448 and USDA-ARS-CRIS project 6612-32000-021-00D. We acknowledge the excellent technical support of Phillip Curry, Melissa Scott, and Dr. James Stanton.